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Prolonged complement activation in mice

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Prolonged complement activation in mice. Because antibody responses to the alternative complement pathway activator, cobra venom factor, are T-dependent and B mice therefore do not develop resistance to its action, it was possible to examine whether renal injury occurs under circumstances of protracted third-phase alternative pathway activation. After periods of up to three months, no evidence from measurements of blood urea or proteinuria or from examinations with light microscopy immunofluorescence or electron microscopy was obtained to indicate a directly nephrotoxic effect of this type of complement activation.

Activation prolongée du complément chez la souris. Du fait que les réponses à l'activateur de la voie alterne du complément, le venin de cobra, sont T-dépendantes et puisque les souris B, de ce fait, ne développent pas de résistance à son action, il a été possible de rechercher la survenue de lésions rénales au cours d'une activation prolongée de la voie alterne. Après des délais atteignant jusqua'à trois mois, aucun argument de néphrotoxicité n'a été tiré des mesures d'urée sanguine et de protéinurie ou de l'étude en microscopie photonique, d'immunofluorescence et électronique.

In this paper, we describe experiments designed to determine whether persistent *in vivo* activation of the alternative pathway of complement (C) directly damages the kidney. Interest in this question has arisen following the recognition that alternative pathway activation in man is a feature of membranoproliferative glomerulonephritis (MPGN), especially the variety characterized by electron dense deposits in glomerular and tubular basement membranes—dense deposit disease [1, 2]. It has been established that activation of the alternative pathway in this disease is due to a serum factor, the so-called C3 nephritic factor (C3-NeF) [3, 4]. Some workers [5–7] claim that this serum factor is related to a normal serum protein involved in physiologic activation of the alternative pathway, but others [8–12] have evidence indicating it to be a distinctive IgG autoantibody. More recently, C3-NeF has been found to be associated with a curious disorder of adipose tissue, partial lipodystrophy, which itself is also sometimes associated with MPGN [13, 14]. It is now established that C3-NeF causes complement activation by binding to and stabilizing the alternative pathway enzymes ($C\overline{3bBb}$ and $C\overline{3bB}$)[a], i.e., prolonging their half-lives [15,16].

So far, studies in experimental animals have failed to provide evidence that systemic activation of complement is nephrotoxic. Verroust, Wilson, and Dixon [17] were unable to produce significant glomerular injury in rabbits by repeated injection of particulate inulin or zymosan, activators of the alternative pathway. It is noteworthy, however, that the degree of complement activation produced in this way is much less than that which occurs in patients with MPGN; furthermore, there are a number of differences between the reaction mechanism of NeF, which works efficiently in the fluid phase by a properdin-independent mechanism [16, 18, 19], acting by making $C\overline{3bBb}$ resistant to its physiologic inactivators [20, 21] and the convertase generated by particulate polysaccharides which require properdin to generate an efficient C3 convertase [22].

Another approach is the use of the complement-activating factor in cobra venom. Cobra venom factor (CVF) causes complement activation by generating a C3 convertase, $\overline{CVF\text{-}B}$, which has a long half-life and, thus, simulates the state brought about by NeF. In acute experiments [23, 24], there was no evidence that CVF caused glomerular injury, but examination of longer-term effects was not possible owing to resistance due to antibody formation, which usually develops within five to ten days. Pryjma and

[a]The suffix "b" after a letter designating the complement protein is applied to the larger fragment produced by cleavage; a bar over the letter or letters indicates an enzymologically active form. $C\overline{3bBb}$ thus consists of the cleavage products of C3, the third component of complement, and the large fragment of factor B of the alternative pathway.

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Humphrey [25] have shown lately, however, that B mice (neonatally thymectomized, lethally irradiated, and bone-marrow-reconstituted mice) fail to make antibodies to CVF, i.e., CVF is a T-dependent antigen. Thus, the effects of protracted complement activation can be studied in such animals. Therefore, we have repeated the experiments of Pryjma and Humphrey to determine whether glomerular damage and, in particular, changes resembling dense-deposit disease develop under these circumstances.

Methods

Animals. Three-to four-week-old CBA mice were anesthetized i.p. with veterinary Nembutal diluted to 1:10 ml/10 g. The skin was divided between the salivary glands, and the plane of connective tissue around the trachea was entered and followed to the sternum. About one-third of the sternum was split, allowing removal of the thymus with a suction pipette. The skin was approximated and pinched together with forceps. Two weeks later, the animals were irradiated with 850 rad from a cobalt source and injected with bone marrow. Marrow from two femurs supplied sufficient cells for three animals. CVF was started some three weeks later.

Cobra venom factor. CVF was purified from crude Naja Naja venom (Sigma) by the method of Ballow and Cochrane [26]. The low-mol-wt active fraction was stored at −70°C. The usual yield of CVF was 24 mg/g of crude venom, and the cobra venom factor had anticomplementary activity, as defined by Ballow and Cochrane, of one unit in each five micrograms of protein.

Plasma C3 depletion. Cobra venom factor was injected i.p. in four equal doses at eight-hour intervals to initiate decomplementation, and thereafter, it was injected as a single dose twice weekly. The dose required to maintain plasma C3 levels at less than 10% of normal was usually four units per mouse (20 μg of CVF). C3 concentrations were assayed by a single radial-immunodiffusion method [27] using a monospecific rabbit anti-mouse C3. The antiserum was raised in rabbits by two injections of zymosan-C3, washed 12 times in 2 M saline [28].

Proteinuria. At intervals, mice were placed in metabolic cages for 24-hr urine collection. Proteinuria was estimated by a Biuret method [29].

Serum urea. Serum urea was measured using a microurease method [30].

Histology. Kidney sections were prepared from one kidney for light microscopy and immunofluorescence, as has been described previously [29]. Electron microscopy was performed on the other kidney. The kidney was perfused *in situ* via the aorta with ice-cold, freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH, 7.2). Thereafter, the tissue was postosmicated, dehydrated, and imbedded in Epon resin.

Animal groups. There were four groups of animals: normal CBA animals injected with saline; thymectomized (TXB) and irradiated CBA animals injected with saline; thymectomized and irradiated CBA animals injected with CVF; and normal CBA animals injected with CVF.

After an initial settling-down period, when blood was taken twice weekly for C3 estimation, complement levels were measured once weekly, before the next injection of CVF. Thus, the C3 levels taken at this time would be expected to be the highest during that week.

Results

Fifteen thymectomized and irradiated mice (TXB mice) were treated with CVF for periods between 72 and 100 days. Two animals died of unidentified pulmonary infection, characterized by alveolar exudation. The other TXB and CVF-treated animals appeared healthy, although they had a smaller weight gain than control animals. The degree of complement depletion is shown in Figure 1, most of the animals having C3 values of less than 10% of normal before the next injection. Only one of the 15 TXB animals developed resistance to CVF; therefore, this animal was excluded. None of the remainder had C3 con-

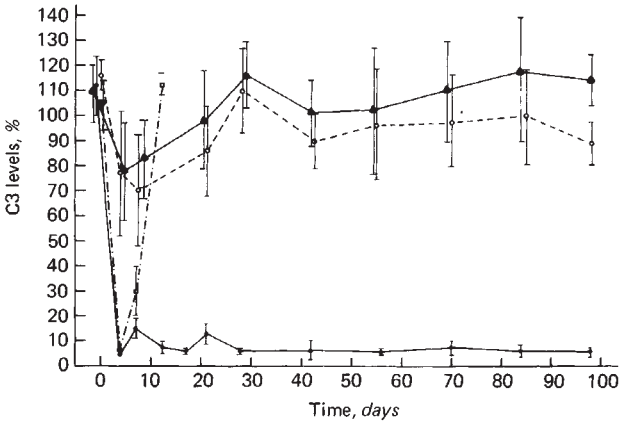

Fig. 1. *C3 levels as a percentage of pooled normal CBA mouse serum. Closed circles* denote thymectomized, irradiated, bone-marrow-protected mice treated with cobra venom factor. *Dashed line with open circles* denote thymectomized, irradiated, bone marrow protected mice injected with saline. *Solid line with squares* denote normal CBA mice injected with saline. *Open squares with alternative dashes and dots* denote normal CBA mice injected with cobra venom factor (only until day 10).

Fig. 2. *An electron micrograph showing a normal capillary loop from a thymectomized, irradiated, bone-marrow-protected CBA mouse after 100 days of decomplementation with cobra venom factor.*

centrations greater than 25% at any measurement during the experiment. Control CBA and TXB animals injected with normal saline had C3 values of 100 ± 40% throughout the experiment. Normal CBA mice injected with CVF developed resistance after five to six days and became totally resistant at ten days.

Serum urea and protein excretion. There was no consistent rise in serum urea or in urine protein excretion in any of the control experimental groups.

Histology. No changes were detected on light microscopy in the kidneys of the control, normal CBA and TXB mice, or in the CVF-treated TXB mice.

Immunofluorescence. Minor deposits of mouse IgG and C3 were found in kidneys from both groups of control animals. TXB CVF-treated animals also had minor deposits of immunoglobulin, but no C3 was detected.

Electron microscopy. Glomeruli from control normal CBA, TXB/CBA mice, and from CVF-treated TXB mice were essentially normal (Fig. 2). A few electron dense deposits were seen in some glomeruli (Fig. 3), but there was no difference between CVF-treated animals and animals given saline.

Discussion

We have been able to confirm the results of Pryjma and Humphrey [15], that in "B mice" it is possible to deplete complement (C) for prolonged periods by repeated injections of CVF, and we have found that after periods of up to three months these animals show no evidence of renal disease: urinalysis, renal function, light microscopy, immunofluorescence, and ultrastructural microscopy were essentially normal. These results indicate that fluid-phase activation of complement via the alternative pathway does not *per se* cause glomerular damage. This is in keeping with our studies of patients with partial lipodystrophy, a condition which is now recognized to be associated with alternative pathway activation [13, 14]. Such patients usually have C3-NeF and hypocomplementemia, but, though prone to develop mesangiocapillary nephritis of the dense-deposit variety, they may not do so. Our findings are also consistent with those of Verroust et al [17], who studied the effects of repeated injection of zymosan and particulate inulin in rabbits, to which we have already referred.

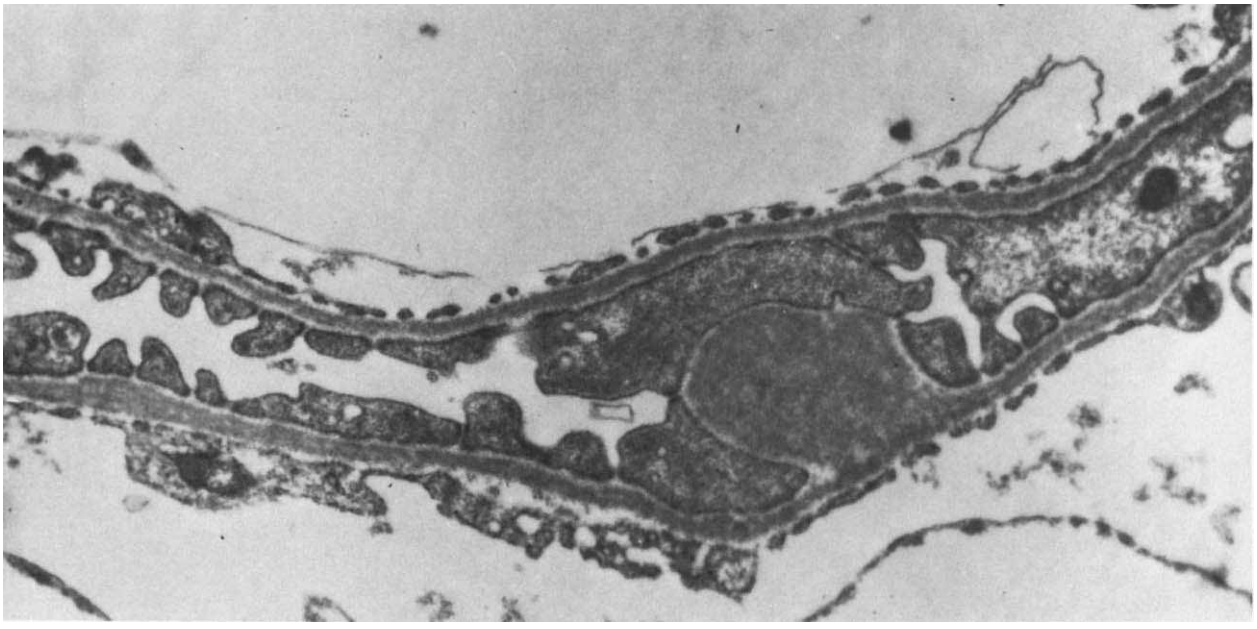

Fig. 3. *An extra-capillary "hump."* These presumed immune deposits were seen infrequently in cobra-venom-treated and control animals.

These clinical and experimental studies indicate that factors other than alternative pathway activation must be involved in the pathogenesis of MPGN, although the clinical data strongly suggest that alternative pathway activation predisposes to its development. We have considered the possibility that longer periods of complement activation might be needed before nephrotoxicity can be recognized; but in patients, electronmicroscopic evidence of recurrence of dense deposit disease in renal allografts is frequently observed within three months of transplant, whereas no comparable damage occurred in this period in our experiments. It is also noteworthy that recurrence of dense deposit disease has been observed lately in the absence of hypocomplementemia [31].

We have previously suggested that the development of nephritis in patients with alternative pathway activation could be a result of complement deficiency, by a mechanism similar to that operating in patients with primary congenital deficiencies of the complement system [13]. An altogether different explanation is that a product of complement activation may be nephrotoxic in certain otherwise predisposed individuals. Our present experiments do not help to distinguish between these suggestions and the mechanisms responsible for the development of renal disease, and, for that matter, the disturbances in adipose tissue remain uncertain.

It is interesting to note that the mortality in these profoundly decomplemented and T-cell deficient animals was relatively low in a normal animal-house environment. Presumably, complement-depleted TXB mice still have defense mechanisms sufficient for the elimination of many bacterial and other infections.

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